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Comparative Structural Requirements of Thirty GRF Analogs for Interaction With GRF- and VIP Receptors and Coupling to Adenylate Cyclase in Rat Adenopituitary, Liver and Pancreas

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ROBBERECHT, P., M. WAELBROECK, D. COY, P. DE NEEF, J.-C. CAMUS AND J. CHRISTOPHE. *Comparative structural requirements of thirty GRF analogs for interaction with GRF- and VIP receptors and coupling to adenylate cyclase in rat adenopituitary, liver and pancreas.* PEPTIDES 7: Suppl. 1, 53–59, 1986.—The ability of 30 synthetic GRF(1–29)-NH_2 analogs to stimulate adenylate cyclase activity was investigated in membranes from rat adenopituitary, rat liver and rat pancreas. In adenopituitary membranes, GRF and GRF analogs interacted with specific GRF receptors, whereas in liver and pancreatic membranes, they interacted with VIP receptors. The C-terminal moiety of GRF was responsible for GRF receptor recognition as the hybrid analog (His^1, $D\text{-}Ala^2$)-GRF(1–9), VIP(10–28) stimulated pituitary adenylate cyclase through the occupancy of VIP receptors only. When GRF or VIP receptors were occupied by GRF analogs, the N-terminal part of the ligand appeared critical for adenylate cyclase activation. This was established by testing 30 GRF analogs mono-, bi- or tri-substituted in positions 1 to 10. Major observations included: (a) the characterization of ($N\text{-}Ac\text{-}Tyr^1$, $D\text{-}Arg^2$)-GRF(1–29)-NH_2 as an antagonist of GRF-stimulated pituitary adenylate cyclase; (b) the discovery of the ($N\text{-}Ac\text{-}Tyr^1$, $D\text{-}Phe^2$)-, (His^1, $D\text{-}Ala^2$, $D\text{-}Ser^3$, $NLeu^{27}$)-, and (His^1, $D\text{-}Ala^2$, $D\text{-}Thr^7$, $NLeu^{27}$)-derivatives of GRF(1–29)-NH_2 as specific antagonists of VIP receptors in rat pancreatic membranes; (c) the importance of the free NH_2 function of amino acid residue 1 for pancreatic adenylate cyclase activation, and (d) the decreased efficiency of iodinated (Tyr^1)-GRF(1–29)-NH_2 as opposed to the non iodinated form, in all systems tested.

GRF VIP Adenylate cyclase Rat pituitary Rat liver Rat pancreas

GROWTH hormone-releasing factors (GRFs or somatocrinins) were first isolated from human pancreatic tumors [7, 14, 21] and later on from porcine and bovine hypothalamus [1,6]. These peptides are structurally related to the VIP/PHI/secretin/glucagon family. On the basis of amino acid composition, rat hypothalamic GRF is somewhat more VIP-like as it possesses a histidine residue in position 1 [22]. Although in man a 44 amino acid form appears to be preferentially processed from a large GRF precursor, synthetic GRF(1–29)-NH_2 is the shortest peptide still endowed with full biological activity [12, 14, 26].

GRF recognizes VIP receptors in human and rat intestinal cells [9], in guinea pig pancreatic acini [13], and also in rat pancreatic plasma membranes [24]. However, its only known physiological effect is to stimulate the release of growth hormone from the anterior pituitary [2] through adenylate cyclase activation and increased cyclic AMP level [5,10].

GRF analogs are synthesized for several uses: (a) to

ABBREVIATIONS

GRF	human growth hormone-releasing factor
VIP	vasoactive intestinal peptide
Kact	concentration required for half-maximal adenylate cyclase activation
I.A.	intrinsic activity

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TABLE 1

ACTIVATION OF ADENYLATE CYCLASE FROM RAT ANTERIOR PITUITARY HOMOGENATES, CRUDE LIVER MEMBRANES AND PANCREATIC PLASMA MEMBRANES BY HUMAN GRF (1–29)-NH_2, 30 GRF ANALOGS AND VIP

Peptide Tested	Anterior Pituitary Homogenate		Liver Membranes		Pancreatic Plasma Membranes	
	Kact (μM)	I.A.	Kact (μM)	I.A.	Kact (μM)	I.A.
GRF(1–29)-NH_2	0.13	1.00	0.70	1.00	1.00	1.00
(D-Tyr^1)-GRF (1–29)-NH_2	0.20	0.98	0.30	1.10	0.65	0.80
(D-Ala^2)-	0.06	0.95	0.15	1.22	0.70	1.50
(D-Asp^3)-	0.16	0.85	1.00	0.88	1.60	0.22
(D-Ala^4)-	0.70	0.85	1.00	0.80	1.80	0.32
(D-Phe^6)-	1.50	0.35	—	0	—	0
(D-Thr^7)-	0.70	0.77	—	0	—	0
(D-Asn^8)-	0.10	0.88	0.80	0.84	1.50	0.70
(D-Ser^9)-	0.15	0.85	0.70	0.84	1.70	0.65
(D-Tyr^{10})-	0.13	0.88	0.50	0.84	1.50	0.50
(Phe^1)-	0.30	0.30	0.30	1.00	1.00	1.00
(Phe^4)-	0.50	0.98	0.30	1.00	0.56	1.70
(pCl-Phe^6)-	0.50	0.82	1.00	0.30	>3.00	0.12
(N-Ac-Tyr^1)-	0.24	0.95	0.30	1.00	1.00	0.21
(N-Ac-Tyr^1, D-Ala^2)-	0.20	0.95	0.30	1.05	0.50	0.34
(N-Ac-Tyr^1, D-Arg^2)-	—	0	1.00	0.45	3.00	0.05
(N-Ac-Tyr^1, D-Phe^2)-	0.50	0.10	2.00	0.20	—	0
(N-Ac-Tyr^1, Trp^6)-	0.70	0.48	1.00	0.53	3.00	0.06
(N-Ac-D-Tyr^1, D-Ala^2)-	0.20	0.90	0.12	1.33	0.30	1.20
(N-Ac-D-Tyr^1, D-Ala^2, D-Asp^3)-	0.30	0.90	0.52	1.33	1.00	0.60
(D-Ala^2, NLeu^{27})-	0.08	0.95	0.25	1.12	1.00	1.50
(His^1, D-Ala^2, NLeu^{27})-	0.14	0.97	0.30	1.30	1.00	1.45
(N-Ac-His^1, D-Ala^2, NLeu^{27})-	0.16	0.75	0.30	0.72	1.00	0.31
(His^1, D-Ala^2, D-Ala^4, NLeu^{27})-	0.93	0.70	0.20	1.30	1.00	1.25
(His^1, D-Ala^2, D-Ser^3, NLeu^{27})-	0.80	0.55	—	0	—	0
(His^1, D-Ala^2, D-Thr^7, NLeu^{27})-	1.20	0.50	3.0	0.60	—	0
(D-Ala^2, D-Asp^3, D-Asn^8, NLeu^{27})-	0.15	0.85	1.5	0.60	>3.0	0.15
(D-Asp^3, D-Asn^8, NLeu^{27})-	0.15	0.85	0.30	1.00	3.0	0.60
(Tyr^1 I_2, D-Ala^2, NLeu^{27})-	1.00	0.35	1.80	0.84	>3.00	0.30
(His^1, D-Ala^2)-GRF(1–9), VIP(10–28)	0.30	0.46	0.001	1.33	0.03	3.00
VIP(1–28)	0.30	0.50	0.0003	1.33	0.03	3.20

Values of Kact and intrinsic activity (I.A.) were calculated from the mean of at least three dose-effect curves performed in duplicate.

search for more potent and/or stable molecules for therapeutic purpose; (b) to obtain an analog that could be easily radioiodinated without losing its biological properties in order to serve as a tracer in radioimmunoassays and receptor assays; (c) to search for antagonists retaining significant affinity for the receptor.

An important challenge in this field is to synthesize analogs conserving GRF specificity for GRF receptors. In the present study we compared the ability of GRF(1–29)-NH_2 and 30 GRF analogs to stimulate or inhibit adenylate cyclase activity in homogenates from rat anterior pituitary, crude rat liver membranes, and purified rat pancreatic plasma membranes that all possess well defined GRF, VIP and/or secretin receptors [15, 16, 18, 25].

METHOD

Peptides

All the peptides used in the present study were synthesized by the solid phase technique [3]. The in vivo and in vitro stimulatory effects exerted by some of these peptides on growth hormone release and quoted in the present study have been reported elsewhere [4, 8, 11].

Adenylate Cyclase Determination and Preparation of Membranes

Adenylate cyclase activity was determined according to the procedure of Salomon *et al.* [19] with minor modifica-

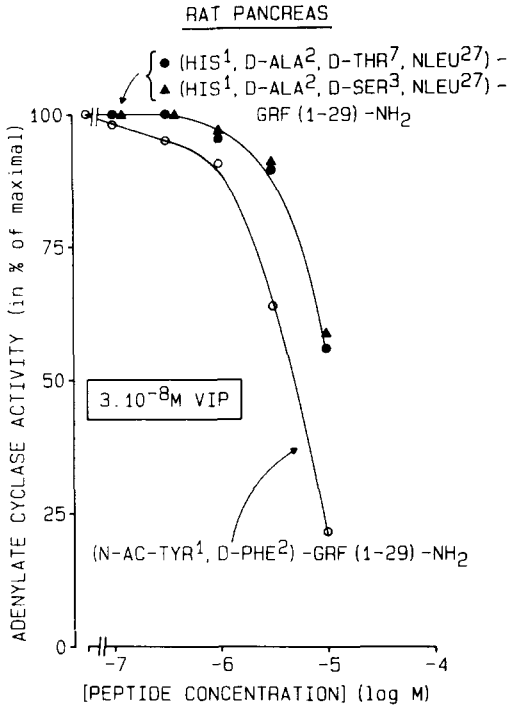

FIG. 1. Inhibition of VIP-stimulated rat pancreatic adenylate cyclase by increasing concentrations of (N-Ac-Tyr¹, D-Phe²)-GRF(1–29)-NH_2 (○), (His¹, D-Ala², D-Ser³, NLeu²⁷)-GRF(1–29)-NH_2 (▲) and (His¹, D-Ala², D-Thr⁷, NLeu²⁷)-GRF(1–29)-NH_2 (●). The results were expressed in % of the value observed in the presence of 30 nM VIP only and were the mean of at least 3 experiments performed in duplicate.

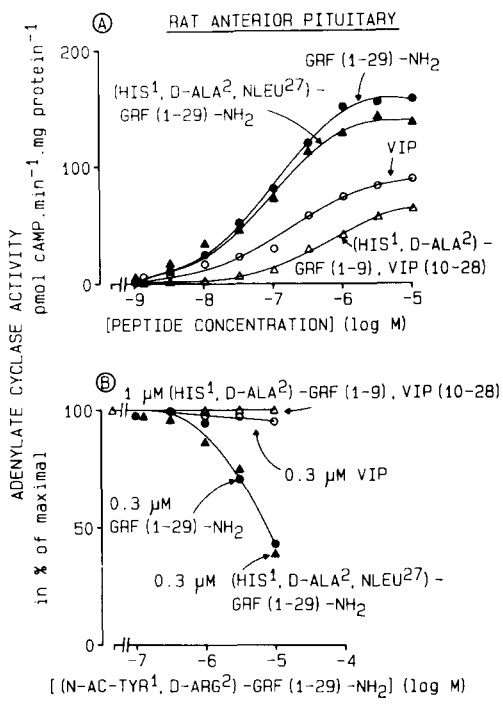

FIG. 2. (A) Dose-effect curves of adenylate cyclase activation in rat anterior pituitary homogenates by GRF(1–29)-NH_2 (●), (His¹, D-Ala², NLeu²⁷)-GRF(1–29)-NH_2 (▲), VIP (○) and (His¹, D-Ala²)-GRF(1–29), VIP(10–28) (△). The results were expressed in pmoles of cyclic AMP produced·min^{-1}·mg $protein^{-1}$ after subtraction of the basal unstimulated activity (48±5, mean±SEM of 3 determinations), and were the mean of 3 experiments performed in duplicate. (B) Inhibition of rat anterior pituitary adenylate cyclase stimulated by 0.3 μM GRF(1–29)-NH_2 (●), 0.3 μM (His¹, D-Ala², NLeu²⁷)-GRF(1–29)-NH_2 (▲), 0.3 μM VIP (○) and 1 μM (His¹, D-Ala²)-GRF(1–9),VIP(10–28) (△) by increasing concentrations of (N-Ac-Tyr¹, D-Arg²)-GRF(1–29)-NH_2. The results were expressed in % of the value observed in the presence of the 4 stimulatory peptides only and were the mean of 3 experiments performed in duplicate.

tions [16]. The reaction started with the addition of either anterior pituitary homogenate, crude liver membranes or purified pancreatic membranes.

Anterior pituitary homogenates were prepared as follows: glands from male adult rats were stored in liquid nitrogen until use; after thawing, they were homogenized with a glass-Teflon pestle homogenizer in 20 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 2.5 mM $MgCl_2$. The homogenate (±1.5 mg protein/ml) was immediately used for adenylate cyclase determination (10 μl homogenate/assay). Crude hepatic membranes were prepared from fresh rat liver as previously described [25]. Ten μl of the membrane suspension (5.0 mg protein/ml) were used in each assay. Rat pancreatic membranes were prepared according to Svoboda *et al.* [23] but β-mercaptoethanol was omitted from all buffers [15]. The membranes were stored in liquid nitrogen at a protein concentration of 1.0 mg/ml. After thawing, 10 μl aliquots were used per assay. It was verified that adenylate cyclase activity was linear during the time period tested and proportional to the amount of protein present in the incubation medium, in all experimental conditions used.

RESULTS

The list of analogs tested, their apparent affinity and their efficacy for adenylate cyclase activation are given in Table 1. Kact represents the peptide concentration required for half-maximal enzyme activation. The intrinsic activity I.A. represents the relative efficacy of the peptide as compared to that of 10 μM GRF(1–29)-NH_2. These values were obtained by analysis of complete dose-effect curves. GRF and GRF analogs were active over a concentration range of two logarithms on liver and pancreatic membranes, and of usually three logarithms on anterior pituitary membranes.

Among the molecules tested, 6 were inactive in at least one system. (D-Phe⁶)-, (D-Thr⁷)-, (His¹, D-Ala², D-Ser³, NLeu²⁷)-GRF(1–29)-NH_2 were devoid of activity on liver and pancreatic membranes. (N-Ac-Tyr¹, D-Phe²)- and (His¹, D-Ala², D-Thr⁷, NLeu²⁷)-GRF(1–29)-NH_2 were inactive on rat pancreatic adenylate cyclase only. (N-Ac-Tyr¹, D-Arg²)-GRF(1–29)-NH_2 was unable to stimulate anterior pituitary adenylate cyclase activity but retained a significant stimulatory effect on liver and pancreatic adenylate cyclase.

All these inactive analogs were tested in combination with GRF(1–29)-NH_2 or VIP to test their potential inhibitory action. In pancreatic membranes, (N-Ac-Tyr¹, D-Phe²)-, (His¹, D-Ala², D-Ser³, NLeu²⁷)-, (His¹, D-Ala², D-Thr⁷, NLeu²⁷)-, (D-Phe⁶)- and (D-Thr⁷)-GRF(1–29)-NH_2 inhibited dose-dependently the VIP-stimulated adenylate cyclase (Fig. 1 and [24]). The Ki values were 1.0, 3.0 and 3.0 μM for, re-

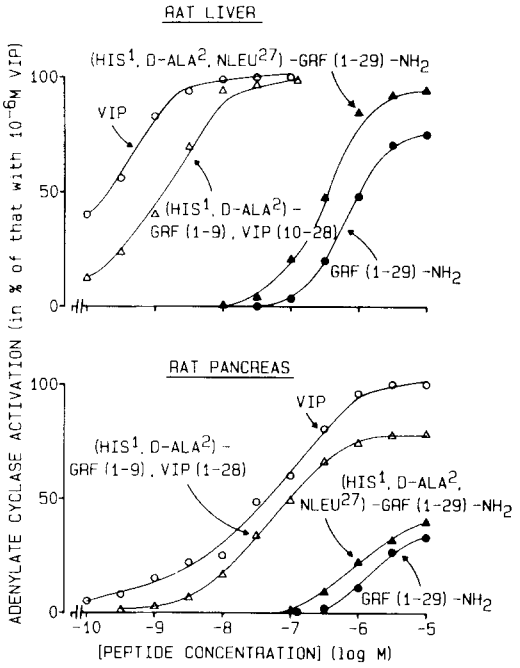

FIG. 3. Dose-effect curves of adenylate cyclase activation of rat hepatic (upper panel) and pancreatic (lower panel) membranes by GRF(1–29)-NH_2 (●), (His¹, D-Ala², NLeu²⁷)-GRF(1–29)-NH_2 (▲), VIP (○), and (His¹, D-Ala²)-GRF(1–9), VIP(10–28) (△). The results were expressed in % of the maximal activity observed in the presence of 10^{-7} M VIP and were the mean of three experiments.

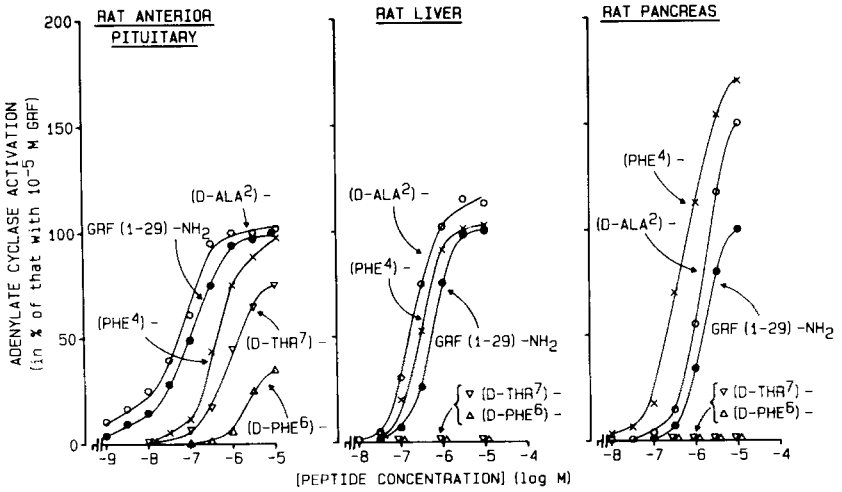

FIG. 4. Dose-effect curves of adenylate cyclase activation in rat anterior pituitary homogenates (left panel), rat liver membranes (middle panel), and rat pancreatic membranes (right panel) by GRF(1–29)-NH_2 (●), (D-Ala²)-GRF(1–29)-NH_2 (○), (Phe⁴)-GRF(1–29)-NH_2 (×), (D-Phe⁶)-GRF(1–29)-NH_2 (△) and (D-Thr⁷)-GRF(1–29)-NH_2 (▽). The results were expressed in % of the values observed in the presence of 10^{-5} M GRF(1–29)-NH_2 and were the mean of 3 determinations performed in duplicate.

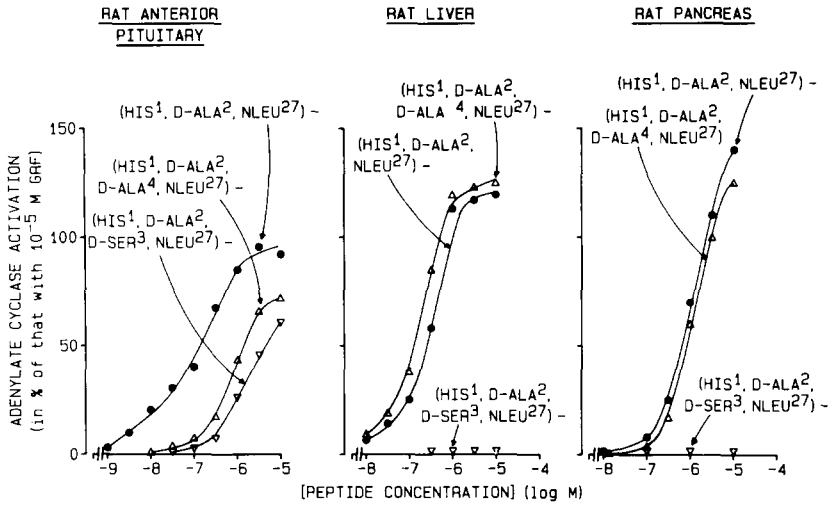

FIG. 5. Dose-effect curves of adenylate cyclase activation of rat anterior pituitary homogenates (left panel), rat liver membranes (middle panel), and rat pancreatic membranes (right panel) by (His¹, D-Ala², NLeu²⁷)-GRF(1–29)-NH_2 (●), (His¹, D-Ala², D-Ala⁴, NLeu²⁷)-GRF(1–29)-NH_2 (△) and (His¹, D-Ala², D-Ser³, NLeu²⁷)-GRF(1–29)-NH_2 (▽). The results were expressed in % of the value observed in the presence of 10^{-5} M GRF(1–29)-NH_2 (not represented on the Figure) and were the mean of three experiments performed in duplicate.

spectively, (N-Ac-Tyr¹, D-Phe²)-, (His¹, D-Ala², D-Thr⁷, N-Leu²⁷)- and (His¹, D-Ala², D-Ser³, NLeu²⁷)-GRF(1–29)-NH_2. The Ki values could not be determined with accuracy for the (D-Phe⁶)- and (D-Thr⁷)-GRF(1–29)-NH_2 analogs but appeared to be higher than 3.0 μM. Of major interest was the fact that the five analogs inhibited GRF(1–29)-NH_2-stimulated pancreatic adenylate cyclase, with the same potency, demonstrating that GRF and VIP acted through the same VIP receptors in that tissue.

We were unable to observe a significant inhibition of rat hepatic VIP- and GRF-stimulated adenylate cyclase activity, when adding the three GRF analogs devoid of stimulatory activity at a 10 μM concentration (the highest dose tested), suggesting a poor affinity of these molecules for hepatic receptors. Evidence derived from binding studies, using ^{125}I-VIP and ^{125}I-helodermin as tracer suggested, however, that GRF and VIP interacted with the same VIP liver receptors and that this interaction was responsible for adenylate cyclase activation [17].

(N-Ac-Tyr¹-D-Arg²)-GRF(1–29)-NH_2 inhibited the GRF-stimulated adenylate cyclase of anterior pituitary membranes dose-dependently and competitively (Fig. 2B). The apparent Ki was around 1 μM and this inhibition was specific as the GRF-analog, used at a 10 μM concentration, was unable to inhibit significantly the VIP-stimulated adenylate cyclase. This suggests that VIP and GRF receptors represented distinct entities with stringent selectivity in rat pituitary so that no interaction of VIP with GRF receptors and of GRF with VIP receptors could be detected in our adenylate cyclase stimulation studies. The specificity of GRF receptors has already been documented in binding studies [20]. There results that our data reflected the interaction of GRF analogs with VIP receptors in hepatic and pancreatic membranes on the one hand, and with GRF receptors in anterior pituitary membranes on the other hand.

The main structural characteristics in the ligands, that were able to influence VIP or GRF receptor occupancy and adenylate cyclase activation, were as follows:

(a) The second moiety of the GRF molecule (amino acids 10 to 29) was essential for GRF receptor recognition: in fact, (His¹, D-Ala²)-GRF(1–9), VIP(10–28)), a hybrid made of a highly favorable modification of the N-terminal moiety of GRF and by the second moiety of VIP, stimulated rat pituitary adenylate cyclase with a dose-effect curve similar to that of VIP (Fig. 2A). This effect was not inhibited by the GRF antagonist (N-Ac-Tyr¹, D-Arg²)-GRF(1–29)-NH_2 (Fig. 2B). In rat liver and pancreatic membranes, the same hybrid was almost as potent as VIP (Fig. 3).

(b) Changes in position 2 were relatively non specific: (D-Ala²)-GRF(1–29)-NH_2 was a superagonist while (D-Arg²)- or (D-Phe²)-derivatives were very poor activator or inhibitor in all three systems (Fig. 4 and Table 1). By contrast, changes in positions 3, 4, 6 and 7 discriminated between GRF and VIP receptors (Table 1, Figs. 4 and 5). Replacement of the acidic aspartate residue in position 3 by the D-isomer of serine suppressed adenylate cyclase activation through VIP receptors but not through GRF receptors. Substitution of alanine in position 4 by phenylalanine increased both the efficacy and potency of the GRF derivative on VIP receptors, but decreased significantly its potency when interacting with GRF receptors. In (His¹, D-Ala², NLeu²⁷)-GRF, a molecule already modified in position 1, 2 and 27, a further replacement of alanine in position 4 by its D-isomer decreased the potency on GRF receptors without altering the interaction with VIP receptors but the resulting (His¹, D-Ala², D-Ala⁴, NLeu²⁷)-GRF(1–29)-NH_2 analog displayed a lower Kact for VIP receptors than for GRF receptors. Substitution on phenylalanine 6 and threonine 7 by their D-isomer led to analogs that were inactive on VIP receptors but retained significant activity on GRF receptors (Fig. 4).

The GRF analogs used allowed to discriminate between rat hepatic and pancreatic VIP receptors. The data obtained

with analogs substituted in position 1 and 3 suggested different requirements for optimal activation of hepatic vs. pancreatic VIP receptors: (a) acetylation of the NH_2 function of the N-terminal amino acid (tyrosine as in human GRF or histidine in rat GRF) reduced drastically the intrinsic activity of the peptide derivatives on rat pancreatic membranes, even when further highly favorable substitutions were operated (Table 1); (b) changes in the stereospecificity of aspartate 3 by using the D-isomer in mono or polysubstituted GRF analogs appeared much more unfavorable for the activation of pancreatic as compared to hepatic adenylate cyclase (Table 1).

DISCUSSION

Besides the specific points already mentioned, some general considerations emerged when comparing the potency and efficacy of 31 peptides on adenylate cyclase stimulation in preparations from rat anterior pituitary, liver and pancreas.

GRF activated adenylate cyclase through interaction with specific GRF receptors in anterior pituitary. We were unable to demonstrate any significant participation of VIP receptors to that stimulation as well as any significant interaction of VIP with GRF receptors. These results agree with the recent characterization of binding sites for radiolabelled GRF on rat anterior pituitary cells [20].

GRF activated adenylate cyclase through interaction with VIP receptors in hepatic and pancreatic membranes. We could not find any evidence for the presence of specific GRF receptors in those tissues [17,24]. This is in line with the results obtained by others on guinea pig pancreas [13] and intestinal cells [9]. Definite proof of the absence of GRF receptors requires, however, the search for specific binding sites using a high affinity radiolabelled GRF analog.

In our experimental conditions, GRF(1–29)-NH_2 showed an affinity that was 5- to 7-fold higher for GRF receptors than for VIP receptors in the 3 tissues tested. It is of practical interest, however, to note that the GRF analogs (Phe^1)-, (Phe^4)-, (N-Ac-D-Tyr^1, D-Ala^2)-, and (His^1, D-Ala^2, D-Ala^4, $NLeu^{27}$)-GRF(1–29)-NH_2 were as potent (or more) when interacting with VIP receptors rather than with GRF receptors, suggesting that amino acids in position 1 and 4 were involved in the specificity of receptor recognition.

Also of practical interest is the fact that substitution of methionine^{27} by Norleucine did not modify the biological properties of the GRF molecule, neither when interacting with GRF receptors, nor when interacting with VIP receptors. Along the same line, we observed that iodination of Tyr^1 gave a very poor analog in all systems tested making it clearly unsuitable for receptor identification in binding studies. By contrast the (His^1, $NLeu^{27}$)- GRF analog could be an excellent candidate in binding studies by avoiding the oxidation of Met^{27} and the iodination of Tyr^1. It is clear, however, that the search for a good radioligand must take into account potential changes in selectivity: for instance, the high affinity analog (His^1, D-Ala^2, $NLeu^{27}$)-GRF(1–29)-NH_2 was less specific than GRF(1–29)-NH_2 when considering its ability to stimulate adenylate cyclase in membranes from anterior pituitary and liver.

The nature of the amino acid and the chemical function present in position 1 appeared to be critical for VIP receptor occupancy on pancreatic membranes: replacement of histidine in position 1 by phenylalanine as well as the acetylation or methylation of His^1 reduced the efficacy of VIP [16]. For GRF analogs, the nature of the first amino acid was, however, of reduced importance on the same model: analogs with Tyr^1, Phe^1, His^1 were equipotent (Table 1). The acetylation of the free NH_2 group decreased drastically the peptide efficacy but this could not be attributed solely to a change in the charge of the molecule as (N-Ac-D-Tyr^1, D-Ala^2)-GRF(1–29)-NH_2 acted as a superagonist.

Finally, it is difficult to predict the biological activity of a polysubstituted analog: replacement of Asp^3 or Asn^8 by their D-isomer was disadvantageous for adenylate cyclase activation through VIP receptors; the simultaneous replacement of both amino acids by their D-isomer was, however, favorable for liver receptors while making this derivative even worse for pancreatic receptors. Paradoxically, the introduction of a third substitution with D-Ala^2 (which was extremely beneficial when intervening alone) provoked a marked decrease in both intrinsic activity and potency.

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